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METHODS OF DETERMINATION OF HYDROXYPROLINE IN BIOLOGICAL FLUIDS AND THEIR USE IN CLINICAL PRACTICE

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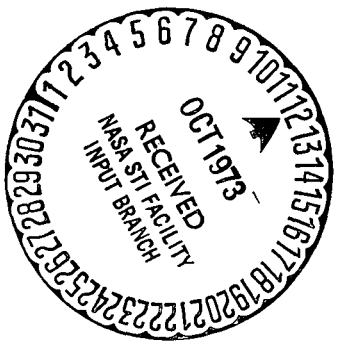

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16. Abstract

A modification of a method for determination of hydroxyproline bound to collagen-like plasma proteins and of total urine hydroxyproline, involving the colorimetric method of Bergman and Locksley, is described. The deviation in parallel tests averaged 2%. Values of both the content of plasma protein hydroxyproline and total urine hydroxyproline of normal individuals are given. A brief account also is given of changes in the above values in collagenous and bone diseases.

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METHODS OF DETERMINATION OF HYDROXYPROLINE IN BIOLOGICAL FLUIDS AND THEIR USE IN CLINICAL PRACTICE

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Collagen is the base of the intercellular matter of connective tissue, and it has an important role in a number of physiological processes. An increase in collagen catabolism and regeneration is an important link in the pathogenesis of many diseases. Twelve-fourteen percent of all amino acid residues of collagen are hydroxyproline [1]. In addition to collagen, this amino acid is present in small quantities in elastin (1-2%), and it is contained in no other proteins of the body, thus being a specific marker of collagen and its decomposition products [1-3]. Therefore, if a collagen metabolism disturbance is accompanied by a transfer of its metabolites to the blood and urine, they can be detected by the hydroxyproline content. /635*

Hydroxyproline is in the free, peptide- and protein-bound forms in human and animal blood [4-6]. By physical-chemical and immunoelectrophoretic properties, blood proteins containing hydroxyproline are analogous to collagen and, in this connection, are called collagen-like plasma protein [6-8]. It contains the principal part (80%) of hydroxyproline and, in the opinion of Le Roy, is more tightly bound to intact collagen fibers than others of its metabolites [9].

The collagen decomposition products in the urine are free (1-3%) and peptide hydroxyproline. The quantity of hydroxyproline eliminated with the urine is higher in persons younger than 20 years, which is determined by growth process [2, 10].

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* Numbers in the margin indicate pagination in the foreign text.

Colorimetric methods of determination of hydroxyproline are based on measurement of the optical density of red chromogen, produced as a result of oxidation and decarboxylation of free amino acids and condensation of the oxidation product with para-dimethyl-aminobenzaldehyde. Hydroxyproline is oxidized with either hydrogen peroxide, according to Neuman and Logan [1], or chloramine T, according to Stegemann [11]. There are a number of deficiencies connected with oxidation by hydrogen peroxide, which can cause unsatisfactory reproducibility of results. Numerous modifications /636 of the method of Neuman and Logan, directed towards elimination of these deficiencies, nevertheless yield to the method of Stegemann, both as to reproducibility of results and in stability of the final color [12-14].

In the work of Bergman and Locksley [14], the effect of all parameters determining the yield and stability of chromogen were studied in the greatest detail. On this basis, the authors proposed a variant of hydroxyproline determination, based on the method of Stegemann. In the proposed method, the stability of the reagents and final color were improved, good reproducibility of results was easily achieved and the technique of analysis was simplified, as well.

The difficulties in hydroxyproline determination in biological fluids are connected with its low concentration and the presence of a number of substances (especially in the blood), interfering with its determination. In the study of normal and pathological collagen metabolism, the choice of method of hydroyxproline determination in plasma is a decisive condition, since errors in the method, arising because of nonspecific plasma components, can considerably exceed the scale of the actual fluctuations in normal and pathological hydroxyproline levels [6, 7, 15]. Thus, the method must be not only very sensitive, but strictly selective. This requires the application of additional operations, directed towards elimination of substances interfering with the colorimetric determination of hydroxyproline from the plasma hydrolyzates.

We carried out a comparative experimental evaluation of the methods of cleansing plasma hydrolyzates of substances which interfere with hydroxyproline determination, as well as methods of colorimetric determination of the latter.

The results of the research carried out served as a basis for development of a modification of the method proposed by Le Roy [7] for determination of hydroxyproline, bound to collagen-like plasma protein. The modification which we propose is based on a combination of chromatographic extraction of hydroxyproline from the plasm hydrolyzates, according to Le Roy [7], with the method of colorimetric determination of it , according to Bergman and Locksley [14].

For determination of total urine hydroxyproline, we use the method of Bergman and Locksley, with preliminary acid hydrolysis of urine and sorption of the pigments with a mixture of activated charcoal and Dowex, according to Prockop and Udenfriend [15].

Reagents and Apparatus

The following reagents are necessary for carrying out the analyses: 1) saturated $Ba(OH)_2$ solution; 2) 12 N, 6 N, 1 N and 0.001 N hydrochloric acid; 3) 6 N sulfuric acid; 4) 1% alcohol solution of phenolphthalein; 5) 70% and 0.35 N NaOH; 6) isopropyl alcohol; 7) absolute ethanol; 8) Dowex 50 x W x 8 ion-exchange resin (200-400 mesh); 9) mixture for precipitation of pigments (Dowex 1 x 8 resin (200-400 mesh) is mixed with finely ground activated charcoal in a 2:1 ratio by weight, washed several times in a Buchner funnel with 6 N hydrochloric acid and dried in a thin layer); 10) oxidizer: a) 7% water solution of chloramine T is prepared extempore, but it can be stored for several weeks in a cold and dark place; b) acetate-citrate buffer, pH 6.0 (57 g sodium acetate, 37.5 g tertiary sodium citrate, 5.5 g citric acid and 385 ml isopropyl alcohol, add distilled water to 1 ℓ. It can

be stored for an unlimited time in a refrigerator. Directly before each series of analyses, the chloramine T solution and buffer solution are mixed in a 1:4 ratio); 11) Ehrlich reagent (para-dimethylaminobenzaldehyde is dissolved in 57% perchloric acid, in the ratio of 2 g aldehyde to 3 ml of acid. The solution can be stored in the refrigerator, in a dark bottle, for a period of several weeks. Immediately before beginning hydroxyproline determination, the solution of aldehyde in perchloric acid is mixed with isopropyl alcohol in a 3:13 ratio); 12) standard hydroxyproline solutions (20 mg hydroxyproline is dissolved in 100 ml of 0.001 N hydrochloric acid. The solution can be stored 1-2 weeks in a refrigerator. Before each series of determinations, working solutions are prepared from the standard hydroxyproline solution (200 µg/ml), with concentrations of 5, 10, 20 and 30 µg/ml); 13) a chromatographic column, 1 x 15 cm in size, is filled to 5 cm with Dowex 50 x W x 8 ion-exchange resin in the H^+ form. Before packing the resin, it is washed successively in a Buchner funnel with distilled water, 6 N NaOH, distilled water and 6 N HCl. /637

All investigations were carried out in a SFD-2 spectrophotometer.

Colorimetric Method of Hydroxyproline Determination

0.2 ml each of neutral or slightly acid solution to be analyzed, containing from 1.5 to 30 µg/ml of hydroxyproline, are poured into a series of test tubes. Then, stirring carefully, 0.4 ml each of isopropyl alcohol and 0.2 ml of oxidizer are added. Three-five min after addition of oxidizer, 2.5 ml each of Ehrlich reagent is poured into every test tube, and they are immersed in a water bath at 60°. After 25 min, the test tubes are transferred to an ice bath and, after cooling, 6.6 ml each of isopropyl alcohol is added. After thorough stirring, the samples are analyzed in the spectrophotometer, in 1 cm cuvettes, at a wavelength of 558 nm, against a control, in which the solution being analyzed is replaced by water.

The color developed practically does not change intensity for a period of 4 h. The hydroxyproline content in each sample analyzed is found from a calibration curve.

Determination of Hydroxyproline Bound to Collagen-Like Plasma Protein

Citrated blood is centrifuged 30 min at 3000-4000 rpm. One ml each of plasma is placed in ampules, in which hydrolysis subsequently is carried out. If the analysis is not conducted on the day the blood is taken, the plasma is stored in a refrigerator at -20°. Our experience shows that storage of plasma samples in the frozen state for a period of more than 5 months does not show an effect on the protein hydroxyproline concentration. Plasma produced with heparin is unsuitable for storage. Four ml of cooled absolute ethanol is added to the ampule, at 4°, containing the samples being analyzed. After 15 min, the alcohol-precipitated proteins are extracted by centrifugation at 4° and 800 rpm for 10 min. The supernatant is carefully poured out and 2 ml of distilled water is poured into the precipitate and stirred. After this, 4 ml of saturated barium hydroxide solution is added. The ampules are sealed and heated at 124° for 16 h. The hydrolyzate is neutralized with 6 N H_2SO_4, by phenolphthalein. Then, 0.2 ml of 6 N hydrochloric acid is added and, after stirring, the barium sulfate is separated out by centrifugation for 10 min at 2000 rpm. The supernatant is transferred to the column with Dowex 50 x W x 8 ion-exchange resin (200-400 mesh), 1 x 5 cm, previously equilibrated with 1 N HCl. The barium sulfate precipitate is washed with 1 ml water and, after centrifuging again, the wash waters also are transferred to the column. After passage of the solution being analyzed through the resin, 5 ml of 1 N HCl is added to each column, and the eluate is discarded. Then, 22 ml of 1 N HCl is added and the eluates containing hydroxyproline are collected in porcelain cups. The collected eluates are evaporated to dryness on a water bath. The dry residue is dissolved in approximately 1 ml of water

and the pH is brought to 6.0 with sodium hydroxide. After this, the contents of the cups are transferred quantitatively to measurement test tubes and the volume is brought up to 4 ml. Subsequently, colorimetric determination of hydroxyproline is carried out by the method described above.

Determination of Total Urine Hydroxyproline /638

Daily urine (3-4 ml of concentrated HCl is used as preservative) is collected from persons receiving food devoid of hydroxyproline, on the second day of the diet. The following were excluded from the ration: meat, fish, gelatin, cream and sweets; proteins were provided by milk, cottage cheese, eggs and cheese. Two ml of daily urine was filtered and hydrolyzed in sealed ampules, with an equal quantity of concentrated HCl, for 3 h at 124°. After completion of hydrolysis, about 0.2-0.3 g of a mixture of activated charcoal and ion-exchange resin was added to the ampules with the hydrolyzates and thoroughly stirred. After 15 min, the contents of the ampule were centrifuged for 10 min at 3000 rpm; 1 ml of the transparent supernatant was transferred to measurement test tubes and neutralized to pH 6.0. The total volume in the measurement test tubes was brought up to 8 ml. Subsequent hydroxyproline determination was carried out by the colorimetric method described above.

Results and Discussion

The original method of determination of hydroxyproline bound to collagen-like plasma proteins, proposed by Le Roy [7], includes the following stages: ethanol precipitation of plasma proteins, alkali hydrolysis of them, extraction of hydroxyproline from substances interfering with its determination by means of column chromatography, and colorimetric determination of hydroxyproline according to Prockop and Udenfriend [15]. The colorimetric method of Prockop and Udenfriend is widely used abroad. However, according

to our data, it does not give sufficiently good reproducibility of results. Firschein and Shill [16] point out this shortcoming. The use of the colorimetric method of Bergman and Locksley [14] in determination of hyroxyproline permitted the reproducibility of results to be improved. Moreover, the method of Bergman and Locksley is simpler in technique than the method of Prockop and Udenfriend, and it requires considerably less consumption of chloramine T. The disagreement between the results of parallel analyses amounts to 2% on the average, and recovery of the standards is 95%. The optical density curve follows the Lambert-Behr law, within the limits of 1-40 μg/ml of hydroxyproline concentration.

In 16 healthy people, ages 21-45 years, examined by us, the content of hydroxyproline bound with collagen-like plasma proteins was 5.7-9.6 μg/ml, and the mean was 7.6 μg/ml. Total hydroxyproline eliminated with the urine amounted to 11.3-36.1 mg/day (19.4 mg/ /day on the average). In this age group, the amount of hydroxy-proline excreted with the urine exceeded 30 mg/day in only one case. In eight juveniles, 16-20 years of age, elimination of hydroxyproline with the urine was 29.8-44 mg/day. The plasma protein hydroxyproline content did not depend on diet or age.

The data which we obtained on content of hydroxyproline bound to collagen-like plasma proteins, in healthy people, corresponds to data published by Le Roy and colleagues [9]. At the same time, the use of the modification proposed by us improves reproducibility of results, simplifies the technique of the method and gives a savings of reagents.

The results reported by K. Trnavsky [17] are understated compared with ours. This is explained by the fact that the method of plasma protein hydroxyproline determination used by K. Trnavsky is based on extraction of collagen proteins with trichloroacetic acid. It is known from the literature [6, 7] that extraction of collagen with trichloroacetic acid is not absolutely quantitative.

Titayev, using a method he proposed [18], did not find protein containing hydroxyproline in healthy children, finding it only in rheumatism patients [4].

The quantities of hydroxyproline eliminated with the urine, /639 which we found in healthy people, correspond to those in the literature [21-23].

Analysis of data obtained by means of the proposed method of determination of hydroxyproline bound to collagen-like plasm proteins and the total urine hydroxyproline, during a dynamic examination of 120 patients with various collagen diseases, permits the conclusion to be drawn that change in the indicators studied makes it possible to decide on the seriousness and extent of collagen metabolism disturbances in various degrees of activity of the pathological process, and, in a number of cases, it can be useful in answering the question of the nosological classification of the disease. We earlier reported in part results of investigations carried out [19, 20].

At the present time, by means of the proposed methods, a study of the plasma protein hydroxyproline content and elimination of hydroxyproline with the urine is being conducted in patients with bone diseases. The most significant changes are observed in excretion of hydroxyproline with the urine in patients with neoplastic and dysplastic diseases (up to 460 mg/day). The preliminary results obtained are evidence of the diagnostic value of hydroxyproline determination in orthopedic practice, which is in agreement with the data of a number of investigators [23-25].

Conclusions

1. A modification is proposed in the method for determination of hydroxyproline bound to collagen-like plasma proteins and the method of determination of total urine hydroxyproline, using the

colorimetric method of Bergman and Locksley. Disagreement between results of parallel analyses amounts to 2% on the average. The advantages of the proposed modification consist of better reproducibility of results, simplification of the techniques of the method and savings in reagents.

2. Study of the blood and urine hydroxyproline content, using the proposed methods, carried out in the collagen disease clinic in pathology of the bones, reflects the degree and extent of the pathological process in connective tissue and, in a number of cases, facilitates nosological determination of a diagnosis.

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